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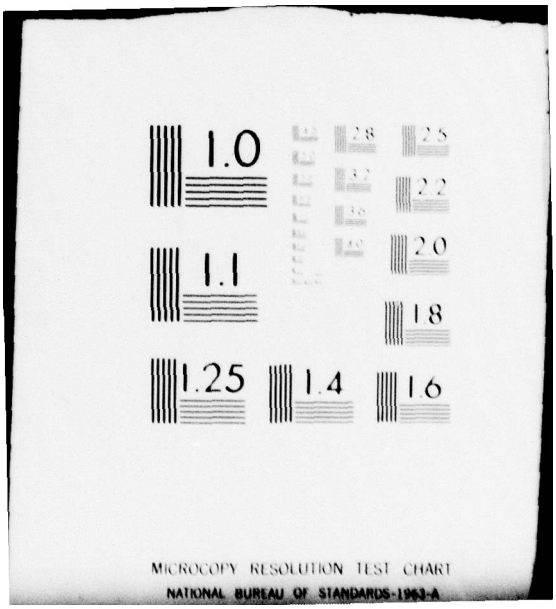
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COMPARATIVE METABOLISM OF PROPELLANT HYDRAZINES.

(10) RONALD C. SHANK, Ph.D.
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AEROSPACE MEDICAL RESEARCH LABORATORY
AEROSPACE MEDICAL DIVISION
AIR FORCE SYSTEMS COMMAND
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TECHNICAL REVIEW AND APPROVAL

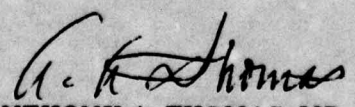
AMRL-TR-79-57

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Information Office (OI) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER



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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Co-administration of hydrazine and ^{14}C-methyl-methionine to rats and mice results in the methylation of liver DNA at the 7-position of guanine; this methylation occurs rapidly and is readily detectable one hour after hydrazine administration. No 7-methylguanine is found in animals given ^{14}C-methyl-methionine alone. Administration of monomethylhydrazine (MMH) also results in the formation of 7-methylguanine in rat and mouse liver DNA but not in DNA from other tissues; MMH serves indirectly as the methyl donor. Administration of 1,1-dimethylhydrazine (UDMH) does not result in DNA

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methylation. The pattern of DNA methylation by hydrazine and MMH was not similar to that produced by 1,2-dimethylhydrazine (SDMH), a strong carcinogen. MMH and UDMH are metabolically oxidized to ~~CO_2~~ carbon dioxide at different rates by various tissues from rats, mice, and hamsters. Studies are in progress to further characterize DNA methylation in animals treated with propellant hydrazines, to determine whether the nitrogen atoms in these hydrazines form adducts with tissue DNA, and to identify all adducts formed by mass spectrometry.

PREFACE

This is the annual report of the subprogram on Comparative Biochemistry and Metabolism and concerns work performed by the Department of Community and Environmental Medicine of the University of California, Irvine on behalf of the Air Force under Contract Number F33615-76-C5005, Work Unit 2312V117. This document describes the accomplishments of the Subprogram from June 1978 through May 1979.

R. C. Shank, Ph.D., served as coordinator for the Subprogram. Acknowledgement is made to M. J. Oldham, L. R. Barrows, R. A. Becker, and D. C. Herron for their significant research contributions and assistance in the preparation of this report. K. C. Back, Ph.D., Chief of the Toxicology Branch, was the technical monitor for the Aerospace Medical Research Laboratory.

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SECTION I

INTRODUCTION

This document constitutes the annual report of the Subprogram on Comparative Metabolism and Biochemistry, and describes the accomplishments of the laboratory from June 1978 through May 1979. The subprogram was established to characterize metabolic pathways of potential Air Force pollutants in selected animal and human tissues which will provide data for estimating whether man metabolizes the compounds at rates and by pathways similar to those for sensitive or resistant animals.

Hydrazine, monomethylhydrazine (MMH), and unsymmetrical dimethylhydrazine (UDMH) are used as rocket propellants and the United States Air Force is currently seeking the safe atmospheric concentrations for these compounds. Under conditions of high concentration and prolonged oral administration, these compounds have produced carcinomas in certain experimental animals.

The Toxic Hazards Research Unit (THRU), operated by the University of California, Irvine, at the Aerospace Medical Research Laboratory at Wright Patterson Air Force Base, Ohio, is currently conducting studies to determine quantitatively the carcinogenicity of hydrazine, MMH, and UDMH under conditions appropriate to practical human exposure. These inhalation carcinogenicity studies are investigating the sensitivity of the mouse, rat, hamster, and dog, recognizing the marked interspecies differences in response to these compounds.

Early studies on the metabolism of these hydrazines are incomplete in that the pathways were not fully defined and metabolism was not considered from the point of view of transformation of the hydrazines to proximate carcinogens. The fact that there are marked differences in the overall oxidation and excretion of hydrazines by various species strongly suggests that the current carcinogenicity studies will demonstrate a variety of responses in tumor production in the four species on test at THRU. The multi-species design provides a better basis for measuring carcinogenicity by reducing the possibility of performing the test in a species uniquely sensitive or resistant and therefore an inappropriate model for man. This design also raises an important question in interpreting the results of the cancer study: which species gives the most accurate prediction of the human response.

Extrapolation from animal data to man is difficult at best, but when carcinogenesis is the consideration, there are no proven measures upon which to base the extrapolation. If an environmental agent requires metabolic activation to generate a proximate carcinogen, which is thought to be the case with the hydrazines, then comparative

metabolic studies on those agents in sensitive and resistant (to tumor formation) species could be useful in predicting which species is the best indicator of the human response, if the metabolism of the agent in man is known. The probable carcinogenicity of the agent precludes in vivo metabolism studies in man, but studies using fresh autopsy material may provide the information needed.

The ultimate objective of the Subprogram on Comparative Metabolism and Biochemistry is to measure the overall rate of metabolism of hydrazine, monomethylhydrazine, 1,1-dimethylhydrazine, and 1,2-dimethylhydrazine in liver, lung, kidney, and large intestine preparations from the mouse, rat, hamster, and human. The formation of several probable metabolites has been postulated for each agent, but these do not appear relevant to the potential carcinogenicity of these compounds; instead, attention is focused on the ability of the hydrazines to bring about alkylation of deoxyribonucleic acids, thus identifying the compounds as indirect alkylating agents. The results of the comparative metabolic studies will be correlated with the results of comparative carcinogenicity studies currently in progress at THRU as an approach to judging human sensitivity to the tumor-producing capabilities of these rocket propellants.

SECTION II
RESEARCH PROGRAM

METHODS

Synthesis of Radiolabeled Methylated Hydrazines

The studies on the comparative metabolism of MMH and UDMH required radiolabeled hydrazines to follow the fate of the methyl groups. For this purpose ^{14}C-MMH and ^{14}C-UDMH were synthesized in the laboratory.

Dimethylsulfate (^{14}C-methyl), 13.3 mCi/mmole was purchased from New England Nuclear (Boston, MA). The dimethylsulfate (2 mCi, 19 mg) was frozen before the vial was opened and 5.5 ul hydrazine were added rapidly to favor the formation of MMH over that of UDMH; 50 ul conc. HCl were added rapidly, with mixing. Water (0.3 ml) was added to bring the final volume to 0.36 ml. The reaction mixture was spotted on thin layer chromatography plates (Avicel 250 microns, Analtech Labs) and reference MMH, UDMH, and SDMH were spotted at one edge of each plate. Plates were developed in isopropanol:water:HCl (165:20:15 or in proportions appropriate to individual batches of plates). The plates were developed until the solvent front had traveled 14.5 cm (about 4 hr.). The side of the plate containing authentic MMH, UDMH, and

SDMH was broken off and sprayed with Folin-Ciocalteau phenol reagent followed by ammonia to locate the positions of each individual hydrazine. MMH and UDMH bands were scraped from the plate and eluted from the cellulose with 3 aliquots of 4 ml water. To the eluate was added 50 ul H_2SO_4, and the solutions were evaporated to dryness in the rotary evaporator at 40°C under vacuum; the radioactive hydrazines were redissolved in water. The yields were 63.7 uCi ^{14}C-MMH (6.65 mCi/mmole) and 134 uCi ^{14}C-UDMH (13.3 mCi/mmole) from 2 mCi dimethylsulfate.

Tritiated MMH and UDMH were synthesized similarly. Five millicuries (0.16 mg) of dimethylsulfate were diluted with stable dimethylsulfate to yield 5.16 mg dimethylsulfate, 122 mCi/mmole. The synthesis produced 228 uCi ^{3}H-MMH (61 mCi/mmole) and 275 uCi ^{3}H-UDMH (122 mCi/mmole).

Liquid Chromatographic Fractionation of DNA Hydrolysates

Several high pressure liquid chromatographic methods have been described in earlier annual reports for the fractionation of DNA hydrolysates into individual pyrimidine and purine bases, their nucleosides, or combinations thereof. Because of the expense of synthesizing radiolabeled MMH and UDMH in the laboratory, efforts were focussed on developing a chromatographic procedure sufficiently sensitive to eliminate the need for radioisotopes. Methylated guanine bases readily fluoresce, while pyrimidine oligonucleotides and adenine fluoresce weakly. Advantage has been taken of these differences in fluorescence in designing a new method for detection of methylated purines in DNA of animals treated with MMH or UDMH. A paper describing the method, described below, has been accepted for publication in Analytical Biochemistry (Herron and Shank, 1979).

Purified DNA is hydrolyzed in 0.1 N HCl (5 mg DNA/ml) at 70°C for 30 minutes to yield pyrimidine oligonucleotides and free purine bases. The hydrolysate is filtered through a 0.65 um filter before injection into the liquid chromatograph (Model 7000B, Micromeritics Corp., Norcross, GA). Fractionation was achieved using a Whatman Partisil-10 strong cation exchange column, 25 cm x 4.5 mm ID and a mobile phase of ammonium phosphate, pH2.0. The concentration of the mobile phase varied from 0.035 to 0.05 M, depending on resolving characteristics of individual columns; optimum concentration of mobile phase was that which permitted separation of 7-methylguanine from adenine (favored by lower concentrations) while still providing an O^6-methylguanine elution peak sufficiently sharp to allow accurate electronic integration (favored by high concentrations). Elution of bases was detected using a variable wavelength fluorescence spectrophotometer (Model LCF-100, Farrand Optical Co., Valhalla, NY) with an

excitation wavelength of 286 nm (max. for 0^6-methylguanine) and emission cutoff at 366 nm. Recording and quantitating of fluorescence peaks were accomplished with a Hewlett Packard Model 3380S reporting integrator (Hewlett Packard Corp., Palo Alto, CA).

The limits of detection in terms of the amount of purine applied to the column are 7 ng for 7-methylguanine and 150 pg for 0^6-methylguanine, which is about 70 times more sensitive than the detection of 0^6-methylguanine by ultraviolet absorption. With these limits of detection, levels of alkylation of DNA as low as 85 umoles 7-methylguanine per mole guanine and 2 umoles 0^6-methylguanine per mole guanine can be detected. This technique has permitted detection of methylated bases in as little as 5 ug DNA from animals treated with strong carcinogens such as 1,2-dimethylhydrazine, dimethylnitrosamine, or methylnitrosourea.

In instances where the level of alkylation of DNA is below detection by fluorescence, radiolabeled toxicant must be used. Liquid chromatographic methods using solutions of inorganic salts as mobile phase present a problem in liquid scintillation counting. For low levels of alkylation large amounts of DNA must be analyzed, by preparative chromatography; this produces large elution peak volumes (often up to 300 ml for 0^6-methylguanine) which must be desalted before counting in scintillation cocktails. A new chromatographic method has been developed which obviates the need to desalt large volumes of mobile phase; DNA hydrolysates can be fractionated on reverse phase columns using aqueous methanol as mobile phase.

DNA hydrolysate is injected onto a Partisil PXS 10/25 ODS-2 column, 25 cm x 4.6 mm ID, and the bases are eluted with a 0-70% methanol in water gradient (pH 4.00) developed linearly over 40 minutes at flow rate of 2 ml/min. Figure 1 is a

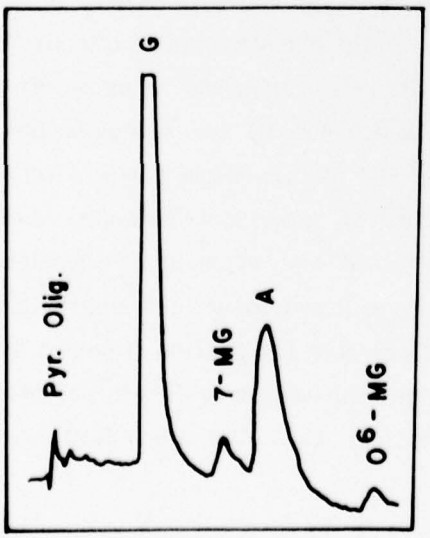


Figure 1. Chromatographic separation of purines and pyrimidine oligonucleotides in DNA hydrolystate using a reverse phase column and fluorescence detector.

typical chromatogram of DNA hydrolysate fractionated by this method. In this case liver DNA was isolated from a rat treated with dimethylnitrosamine and detection of eluted bases was accomplished with a fluorescence detector at an excitation of 286 nm and emission cutoff of 366 nm. Under these chromatographic conditions the pyrimidine oligonucleotides fluoresce only weakly; Table 1 gives the elution volumes for each base identified.

TABLE 1

CHROMATOGRAPHIC (HPLC) FRACTIONATION OF LIVER DNA HYDROLYSATE PREPARED FROM RATS TREATED WITH DIMETHYLNITROSAMINE: METHANOL GRADIENT AND REVERSE PHASE COLUMN

Fraction	Retention Volume, ml
Pyrimidine oligonucleotides	2-6
Guanine	16
7-Methylguanine	27
Adenine	33
0^6-Methylguanine	47

Eluted bases are in an aqueous methanol solution which facilitates evaporation to dryness preliminary to counting the fractions for radioactivity or chemical derivatization for further study.

GC/MS Analysis of DNA Purines and Pyrimidines

Administration of hydrazine to rats and mice results in the formation of a DNA component with liquid and paper chromatography and ultraviolet absorption characteristics identical to those of 7-methylguanine. A method has been developed which should permit confirmation of the identity of this putative base by mass spectrometry. In addition, the method should permit confirmation of the identities of other DNA components and adducts and facilitate studying the interactions between

DNA and the nitrogen atoms in animals treated with ^{15}N-hydrazine. The method involves isolation of the individual base from a DNA hydrolysate by reverse phase high pressure liquid chromatography, silylating the base to increase the volatility of the compound, and analyzing the silylated base by gas chromatography - mass spectrometry.

Reference thymine, cytosine, 5-methylcytosine, uracil, adenine, guanine, 7-methylguanine, and N^6-methyladenine were silylated by a slight modification of the method of Gehrke and Lakings (1971); O^6-methylguanine and O^6-ethylguanine, synthesized according to the method of Balsinger and Montgomery (1960), were also studied. Approximately 300 ug of base in 300 ul of appropriate solvent (e.g. butanol:acetic acid:water, 24:30:50; acetone; water; 0.1 N HCl; guanine, which is only sparingly soluble in most common solvents, was transferred as a suspension) were transferred to a silylation vial and dried under warmed nitrogen; to the vial were added 150 ul acetonitrile and 150 ul BSTFA (bis-trimethylsilyl trifluoracetamide) (not all of the base dissolved). The vials were sealed and heated in a sand bath at 150°C for 30 minutes and then cooled to room temperature.

Within 4 hours of synthesis 2-5 ul of the silylation mixture were injected onto a gas chromatographic column (3% OV-1, 80/100 WHP, 4 ft, Hewlett Packard) for mass spectral analysis. The instrument used was a 5992A GC/MS (Hewlett Packard) and the conditions were as follows: helium flow of 30 ml/minute, injection port temp of 225°, initial column temp of 90° which was held constant for 4 minutes then increased at a rate of 8°/minute to a maximum temperature of 280°. The mass spectrometer was tuned automatically using perfluorotributylamine as the calibrating standard; the instrument scanned the atomic mass unit range of 10 to 500 at a rate of 690 scans per second. The gas chromatographic and mass spectral characteristics for each silylated base are given in Table 2. Individual mass spectra are illustrated in Figures 2-15.

TABLE 2

GAS CHROMATOGRAPHIC AND MASS SPECTRAL CHARACTERISTICS OF SILYLATED PYRIMIDINES AND PURINES

Silylated Base	Elution Time, Min.	Elution Temp, °C	Base Peak	Other Major Peaks (rel. abund.)			
Thymine	12.9	161	255	45(23)	59(21)	73(50)	100(11)
				113(47)	119(12)	140(15)	147(44)
				256(20)	270(35)		

TABLE 2 Continued

Silylated Base	Elution Time, Min.	Elution Temp, °C	Base Peak	Other Major Peaks (rel. abund.)			
Cytosine	14.8	176	73	43(27)	44(11)	45(54)	59(16)
(2 peaks)				70(13)	72(22)	74(16)	98(50)
				99(10)	100(25)	112(29)	125(14)
				147(11)	170(27)	240(45)	254(36)
				255(23)			
	16.6	191	73	45(29)	100(13)	170(12)	197(14)
				312(19)	326(14)	327(13)	
5-Methylcytosine	15.3	180	254	18(24)	28(43)	29(20)	32(16)
				41(32)	43(39)	44(15)	45(40)
				55(27)	56(10)	57(19)	59(19)
				69(19)	70(11)	72(10)	73(90)
				74(19)	75(11)	81(11)	83(10)
				84(12)	91(12)	100(19)	111(12)
				112(28)	120(29)	139(17)	147(26)
				184(16)	255(25)	269(32)	
Uracil	12.3	156	241	28(12)	43(15)	45(40)	73(49)
				99(96)	100(15)	113(52)	126(30)
				131(11)	147(71)	148(12)	242(23)
				255(41)	256(50)	257(13)	
Adenine	19.5	214	264	43(14)	45(27)	73(72)	84(11)
				192(14)	265(22)	279(20)	
Guanine	22.6	239	73	45(30)	75(10)	147(12)	352(41)
				353(13)	367(19)		
7-Methylguanine	23.1	243	294	73(69)	75(19)	180(30)	222(14)
				237(14)	295(24)	296(9)	309(20)
O^6-Methylguanine	21.8	232	222	42(18)	44(17)	58(27)	69(13)
(2 peaks)				73(34)	74(11)	88(61)	99(11)
				190(14)	206(11)	223(20)	237(34)
				294(19)			
	22.4	237	73	45(32)	58(36)	74(22)	84(13)
				89(17)	99(15)	294(84)	295(18)
				309(29)			

TABLE 2 Continued

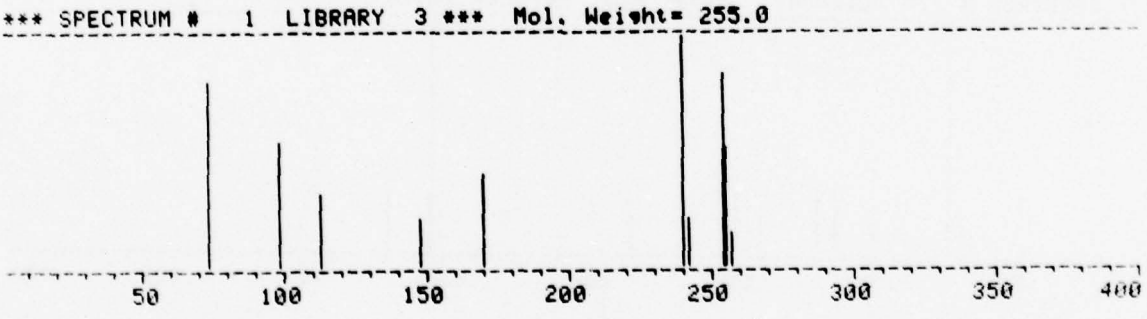
Silylated Base	Elution Time, Min.	Elution Temp, °C	Base Peak	Other Major Peaks (rel. abund.)			
O^6-Ethylguanine	23.1	243	208	18(17)	28(92)	29(20)	32(25)
(2 peaks)				43(20)	45(22)	73(31)	75(30)
				99(17)	166(33)	236(56)	251(40)
	23.8	248	208	29(16)	44(16)	45(29)	69(10)
				73(57)	75(35)	99(19)	166(36)
				236(61)	251(47)	280(31)	323(25)
N^6-Methyladenine	18.4	205	221	15(16)	27(12)	28(86)	29(15)
(2 peaks)				30(44)	42(22)	43(30)	44(17)
				45(29)	53(23)	54(10)	55(11)
				66(21)	67(13)	69(10)	72(13)
				73(43)	84(13)	93(41)	94(11)
				119(22)	120(44)	121(42)	138(14)
				148(25)	149(74)	150(16)	164(19)
				165(34)	192(30)	193(31)	206(58)
				220(25)			
	20.3	220	278	43(11)	45(27)	73(74)	84(12)
				206(18)	220(12)	279(23)	293(14)

Silylated Thymine

Entry	Mass	Abundance
1	45	257
2	73	412
3	113	480
4	120	238
5	140	126
6	147	393
7	255	1000
8	256	202
9	270	441
10	271	111

Figure 2. Mass spectrum of disilylthymine.

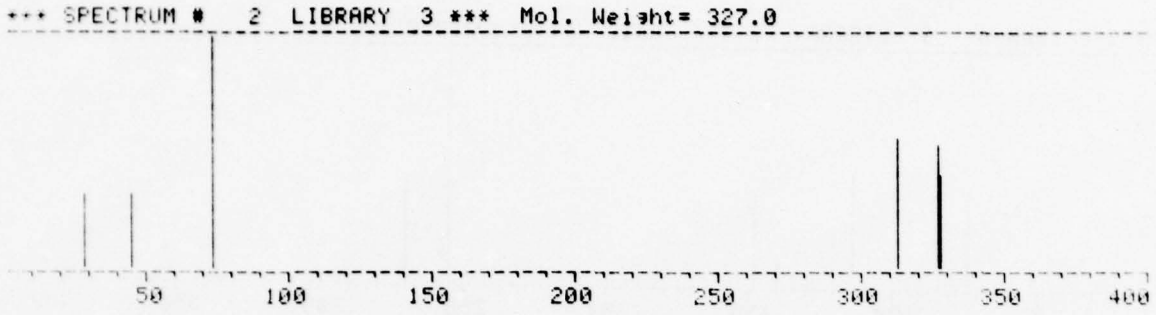
Silylated Cytosine - Fast Peak



Entry	Mass	Abundance
1	73	796
2	98	536
3	112	319
4	147	207
5	170	399
6	240	1000
7	241	206
8	254	828
9	255	514
10	256	146

Figure 3. Mass spectrum of disilylcytosine.

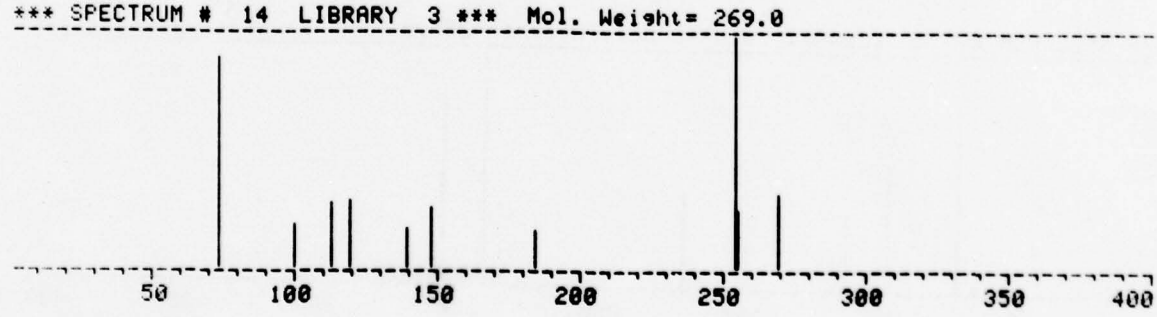
Silylated Cytosine - Slow Peak



Entry	Mass	Abundance
1	28	318
2	45	324
3	73	1000
4	312	561
5	326	534
6	327	397
7	0	0
8	0	0
9	0	0
10	0	0

Figure 4. Mass spectrum of trisilylcytosine.

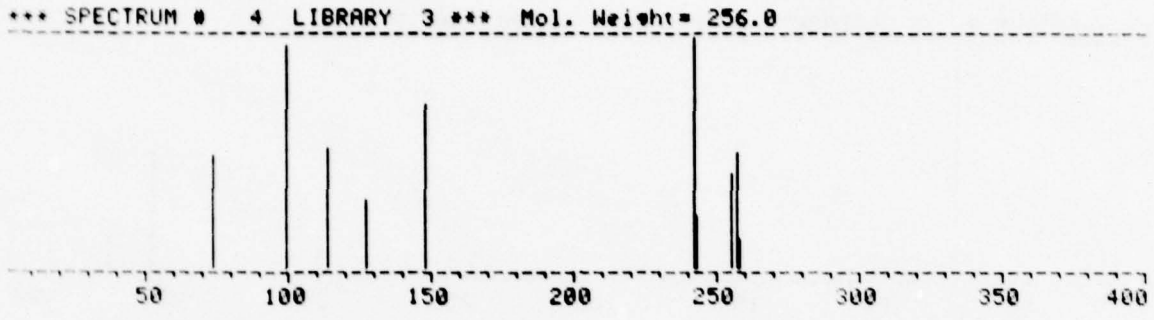
Silylated 5-Methylcytosine



Entry	Mass	Abundance
1	73	899
2	100	191
3	112	281
4	119	292
5	139	172
6	147	259
7	184	156
8	254	1000
9	255	248
10	269	323

Figure 5. Mass spectrum of disilyl-5-methylcytosine.

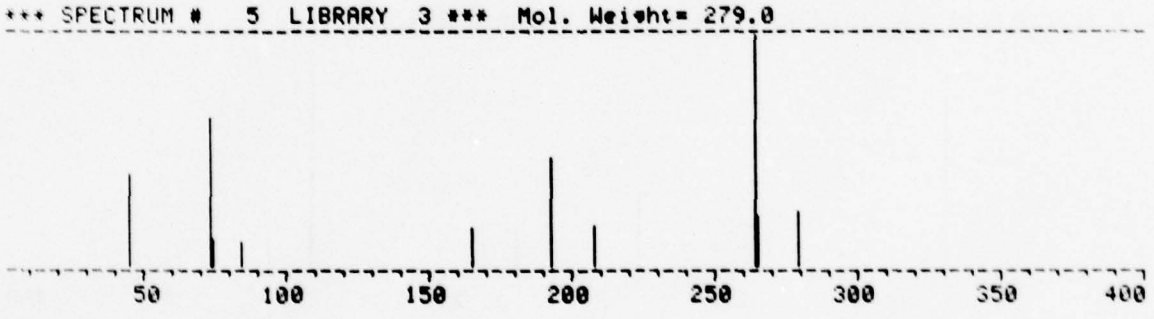
Silylated Uracil



Entry	Mass	Abundance
1	73	488
2	99	957
3	113	524
4	126	303
5	147	709
6	241	1000
7	242	230
8	255	405
9	256	495
10	257	126

Figure 6. Mass spectrum of disilyluracil.

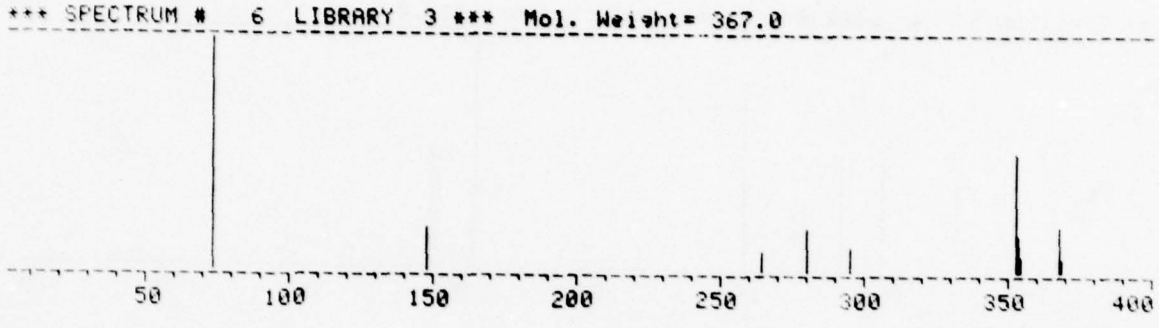
Silylated Adenine



Entry	Mass	Abundance
1	45	397
2	73	644
3	74	122
4	84	111
5	165	169
6	192	469
7	207	178
8	264	1000
9	265	222
10	279	241

Figure 7. Mass spectrum of disilyladenine.

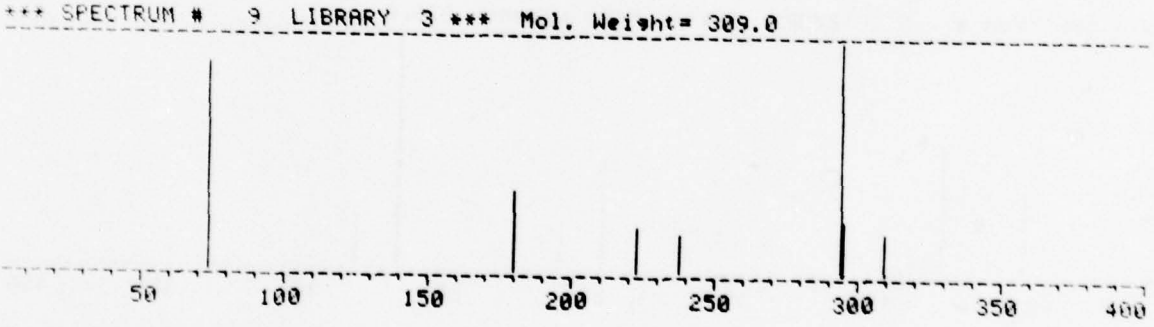
Silylated Guanine



Entry	Mass	Abundance
1	73	1000
2	147	188
3	264	87
4	280	194
5	295	111
6	352	506
7	353	156
8	354	72
9	367	198
10	368	63

Figure. 8. Mass spectrum of trisilylguanine.

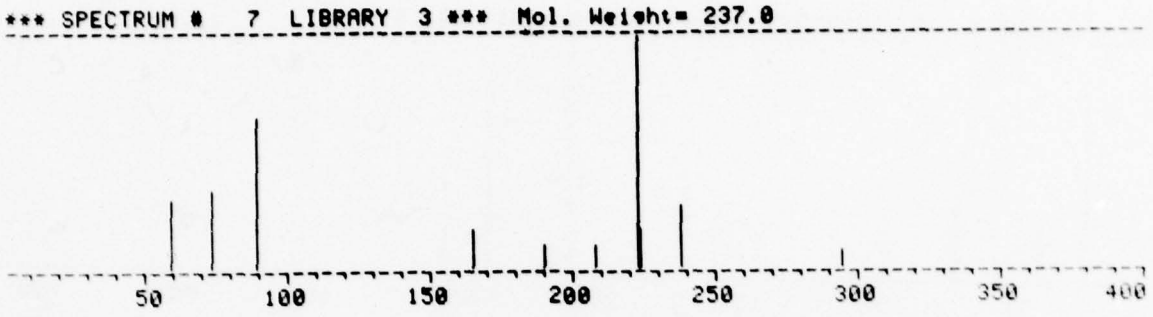
Silylated 7-Methylguanine



Entry	Mass	Abundance
1	73	892
2	180	347
3	222	205
4	237	166
5	294	1000
6	295	228
7	309	183
8	0	0
9	0	0
10	0	0

Figure 9. Mass spectrum of disilyl-7-methylguanine.

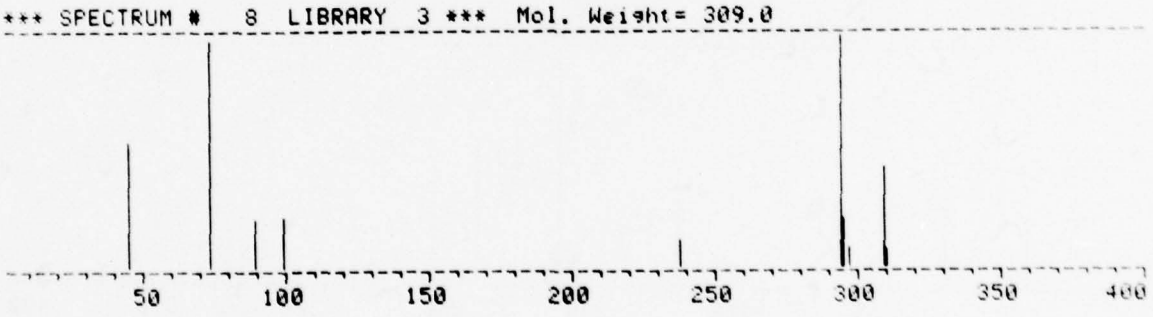
Silylated 0^6-Methylguanine - Fast Peak



Entry	Mass	Abundance
1	59	298
2	73	343
3	89	651
4	165	171
5	190	99
6	207	99
7	222	1000
8	223	167
9	237	274
10	294	84

Figure 10. Mass spectrum of monosilyl-0^6-methylguanine.

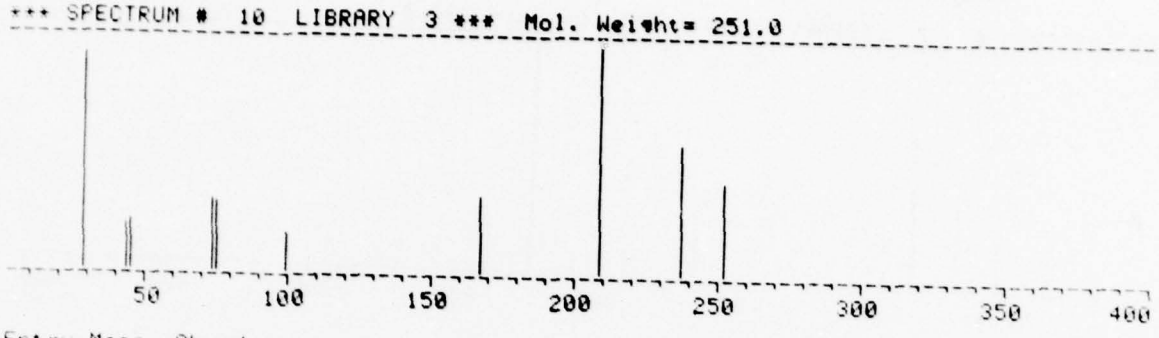
Silylated 0^6-Methylguanine - Slow Peak



Entry	Mass	Abundance
1	45	540
2	73	970
3	89	210
4	99	220
5	237	120
6	294	1000
7	295	210
8	296	90
9	309	430
10	310	80

Figure 11. Mass spectrum of disilyl-0^6-methylguanine.

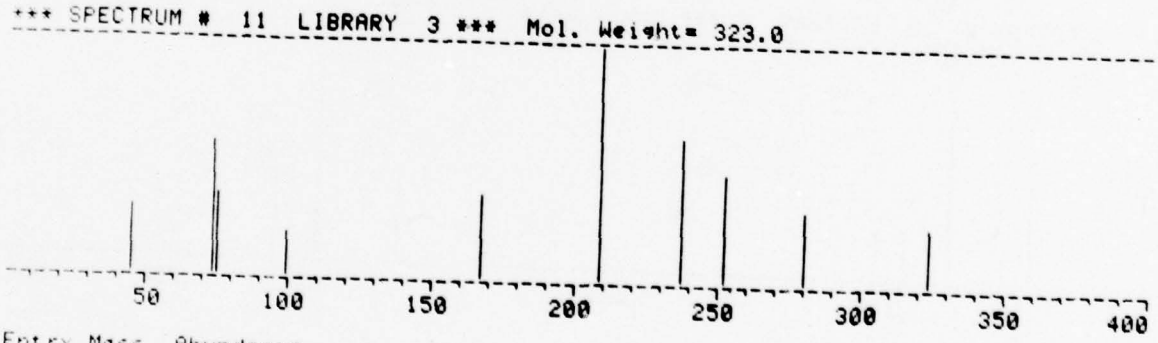
Silylated 0^6-Ethylguanine - Fast Peak



Entry	Mass	Abundance
1	28	920
2	43	200
3	45	220
4	73	310
5	75	300
6	99	170
7	166	330
8	208	1000
9	236	560
10	251	400

Figure 12. Mass spectrum of monosilyl-0^6-ethylguanine.

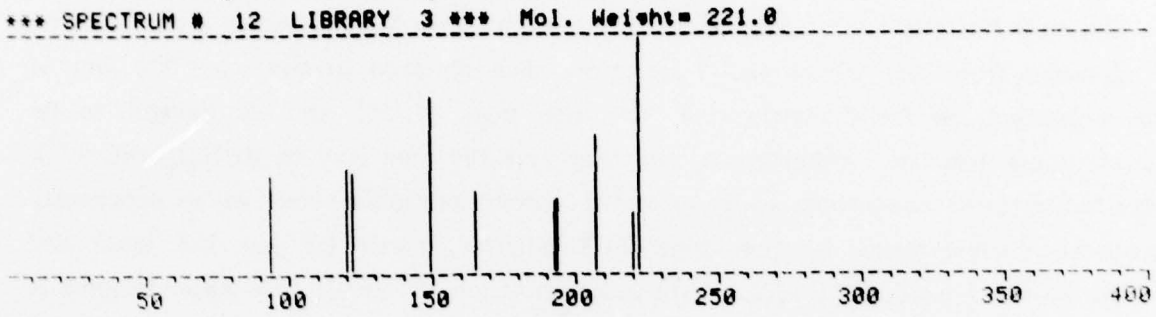
Silylated 0^6-Ethylguanine - Slow Peak



Entry	Mass	Abundance
1	45	290
2	73	574
3	75	350
4	99	191
5	166	360
6	208	1000
7	236	611
8	251	465
9	280	305
10	323	250

Figure 13. Mass spectrum of disilyl-0^6-ethylguanine.

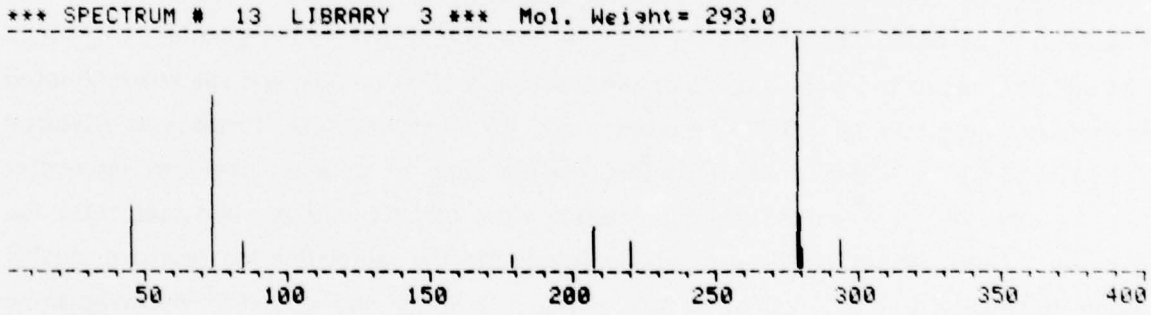
Silylated N^6-Methyladenine - Fast Peak



Entry	Mass	Abundance
1	93	411
2	120	438
3	121	422
4	149	742
5	165	339
6	192	299
7	193	309
8	206	578
9	220	247
10	221	1000

Figure 14. Mass spectrum of monosilyl-N^6-methyladenine.

Silylated N^6-Methyladenine - Slow Peak



Entry	Mass	Abundance
1	45	270
2	73	741
3	84	123
4	178	65
5	206	175
6	220	120
7	278	1000
8	279	228
9	280	87
10	293	135

Figure 15. Mass spectrum of disilyl-N^6-methyladenine.

A few ions are common to all spectra and are due to the silylation of the bases; ion 73 amu is the $(CH_3)_3Si^+$ fragment and ion 147 amu probably represents a combination of elements from two trimethylsilyl moieties. For silylated thymine, ion 270 amu is the molecular ion for disilylthymine, the base peak of 255 amu is thought to be disilylthymine less one methyl group, and fragment 100 amu may be $((CH_3)_2SiNC=0)^+$). Silylated cytosine characteristically gave two chromatographic peaks which apparently represent disilylcytosine (elution time 14.8 minutes, molecular ion 255 amu) and trisilylcytosine (elution time 16.6 minutes, molecular ion 327 amu). The major fragments of disilylcytosine seem to be 240 amu (disilylcytosine less one methyl group), 98 amu $((CH_3)_2SiN-CH=CH)^+$), and 70 amu $((CO-N=C-NH_2)^+)$. For the trisilylcytosine, peak 312 amu presumably is the molecular ion less one methyl group. 5-Methycytosine, on the other hand, apparently formed only the disilylated derivative, 269 amu; the base peak of 254 is probably disilyl-5-methylcytosine less one methyl group. Uracil formed the disilylated derivative 256 amu, and again, the base peak seems to be disilyluracil less one methyl group, 241 amu. In the case of adenine, disilyladenine is the molecular ion, 279 amu, and other characteristic fragments appear to be 264 amu (disilyladenine less one methyl group) and 192 amu (monosilyladenine less one methyl group). The molecular ion for silylated guanine is 367 amu (trisilylguanine) and the molecular ion less one methyl group (352 amu) is a major fragment. Of all the bases silylated so far, 7-methylguanine was the least reactive substrate and yields were considerably less than with other bases. The molecular ion, 309 amu, is disilyl-7-methylguanine; fragments 294 and 237 appear to be the molecular ion less one methyl moiety and the monosilylated derivative, respectively. The alkylated base, 0^6 methylguanine, forms two silylated derivatives: monosilyl-0^6-methylguanine, elution time of 21.8 minutes and molecular ion 237 amu; disilyl-0^6-methylguanine, elution time of 22.4 minutes and molecular ion 309 amu. Both derivatives have as major fragments the molecular ion less one methyl group (monosilyl-0^6-methylguanine less $-CH_3$, 222 amu, and disilyl-0^6-methylguanine less $-CH_3$, 294 amu).

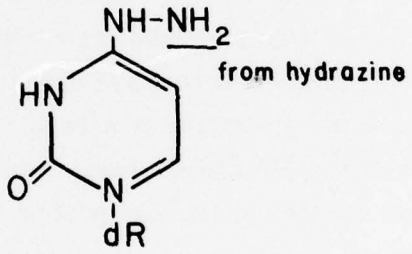
Two silylated derivatives were formed with 0^6-ethylguanine, monosilyl-0^6-ethylguanine with a retention time of 23.1 minutes and the disilyl compound with a retention time of 23.8 minutes. The molecular ion of the monosilyl compound is 251 amu and major fragments are 236 amu and 208 amu, the monosilylated base less a methyl group and less one methyl and one ethyl group, respectively. The disilyl compound has a molecular ion of 323 amu and fragments similarly to the monosilyl compound.

N^6-Methyladenine also formed two silylated derivatives, the monosilyl-N^6-methyladenine, 221 amu, and the disilyl-N^6-methyladenine, 293 amu; both derivatives also had the silylated compound less one methyl group as major fragments: 206 amu for the monosilylated base less one methyl group and 279 amu for the disilylated base less the methyl moiety.

Work is continuing to include 1-methyladenine, 7-ethylguanine, and 7-ethyladenine in this group of silylated bases for mass spectral analysis. Direct application of this method to confirm the identity of suspect 7-methylguanine in liver DNA from hydrazine-poisoned rats and mice is an immediate goal.

Gas Chromatographic Separation of Simple Amines

In the reaction flask hydrazine is known to interact with purines and pyrimidines directly forming hydrazine derivatives; for example, hydrazine and cytosine react at pH 6-8 to form N^4-aminocytosine (Lingens and Schneider-Bernlohr, 1965; Brown et al., 1966).



N^4-aminocytosine

A study is planned to determine the metabolic fate of the nitrogen atoms of hydrazine (and MMH and UDMH) using ^{15}N-labeled material; the extent to which hydrazine is converted to DNA adducts will be determined using mass spectrometry. To accomplish this it has been necessary to develop a method to determine whether any nitrogen from hydrazine binds at all to DNA in vivo.

The current method involves isolating DNA from rats treated with ^{15}N-hydrazine, submitting the DNA to a Kjeldahl digestion to convert the total organic nitrogen to ammonia, and steam distilling the ammonia directly into an excess of ethyliodide, CH_3CH_2I. The ammonia and ethyliodide react to form ethylamine, $CH_3CH_2NH_2$, diethylamine, $(CH_3CH_2)_2NH$, and triethylamine, $(CH_3CH_2)_3N$. The reaction products are then injected into the gas chromatograph - mass spectrometer to separate the amines and measure their $^{15}N/^{14}N$ ratios.

Various conditions for the gas chromatography have been explored. The most successful separations to date have been achieved with a Tenax 60/80 mesh 10'x1/8" stainless steel column in a Varian 1700 gas chromatograph with a flame ionization detector. The GC conditions are: injector temperature 225°, helium carrier gas flow rate 15 ml/min., air flow 200 ml/min., hydrogen flow 15 ml/min., temperature program begins at 90° and increases at 6°/min to a final temperature of 200°, detector temperature, 225°. Monoethylamine and diethylamine elute separately within the first 14 minutes but as triethylamine elutes the solvent begins to come off the column, obscurring the last peak. Use of a higher boiling point solvent, such as n-hexanol may solve this problem; lower molecular weight alcohols all interfere with the elution of the amines. Once the GC conditions are optimized, DNA from ^{15}N-hydrazine-treated rats will be analyzed as outlined above.

HYDRAZINE METABOLISM

The study on hydrazine metabolism has concentrated on a proposed pathway expected to be relevant as far as hydrazine carcinogenicity is concerned. It has been assumed that if hydrazine is a carcinogen, it is active directly or indirectly by chemically modifying DNA in the target cell. Hydrazine is a hepatotoxin and has been reported to cause tumors in the lung of the mouse and the nasal cavity of the rat. Administration of hydrazine to rats and mice resulted in the methylation of liver DNA, and the study of this interaction has been pursued as having possible relevance to the toxicity and carcinogenicity of hydrazine. Although the liver is not the target organ for the carcinogenicity of hydrazine, the liver may serve as an indicator of methylation in other tissues; many chemical carcinogens, which do not induce tumors in the liver, will interact with the most reactive nucleophilic site in liver DNA, the 7-position of guanine, as well as at this and other sites in target organ DNA. Thus, finding 7-methylguanine in DNA in the liver suggests that this and other bases (perhaps more important in carcinogenesis) may form in target tissue. Much effort has been put into defining the conditions under which hydrazine administration can result in methylation of DNA in the liver and other tisses and what significance this might have in carcinogenesis.

Rats are fasted overnight to reduce the hepatic stores of glycogen which interfere with the DNA isolation. Between 8 and 9 a.m., the rats are given orally a solution of hydrazine in 0.1 N HCl (intubation volume approximately 0.1 ml); controls are given 0.1 N HCl only. At the same time and every hour thereafter for four hours, all rats are given ^{14}C-methyl-methionine (approximately 20 uCi) intraperitoneally, and are killed

by decapitation one hour after the last methionine injection. Liver DNA is isolated, acid hydrolyzed, and chromatographically separated into pyrimidine oligonucleotides and free purine bases. At a dose of 60 mg hydrazine/kg body weight (LDO.01), but not at a dose of 30 mg/kg, the presence of 7-methylguanine in the DNA hydrolysate can be detected.

To confirm the identity of 7-methylguanine, 90 mg of liver DNA were prepared from 12 rats given orally 60 mg hydrazine/kg 5 hours before decapitation; methionine was not administered to these animals. The DNA hydrolysate, without added 7-methylguanine, was fractionated by high pressure liquid chromatography as described above, and suspect 7-methylguanine was collected, dried under vacuum, and redissolved in 0.1 ml 0.1 N HCl. This material was spotted on Whatman 3M chromatography paper which was then developed with butanol:ammonium hydroxide:water (85:2:12) according to Singer and Frankel-Conrat (1975). Two ultraviolet absorbing spots were resolved with retention volumes corresponding to those of adenine and 7-methylguanine (7-methylguanine elutes immediately after adenine in the column chromatography method). The suspect 7-methylguanine was eluted from the paper with 0.8 ml 0.1 N HCl. The ultraviolet absorption spectrum of this eluate was determined at pH 1 and then at pH 11; these spectra closely followed those of reference 7-methylguanine under the same conditions. Using a molar extinction coefficient of $8.97 \times 10^{-3} M^{-1} cm^{-1}$ for 7-methylguanine, the amount of this base was estimated to be 82 umoles 7-methylguanine per mole guanine isolated from the DNA hydrolysate.

This level of 7-methylguanine in DNA is at the limit of detection for the newly developed fluorescence method described above. Several attempts have been made to measure the amount of 7-methylguanine in DNA hydrolysates by the fluorescence method but they have not been successful. Occasionally, O^6-methylguanine was detected in hydrolysates of mouse liver DNA using the radioactive methionine procedure but this base has not been detected using the fluorescence technique. All further studies, then, have relied on labeling the methylating intermediate by giving animals radioactive methionine. To reduce the cost of the studies ^{3}H-methyl-methionine has been substituted for the ^{14}C-labeled amino acid.

The methylation of liver DNA in rats occurs shortly after oral administration of the hydrazine. Male Fischer 344 rats, 112-195 g body weight, were given at time zero 60 mg hydrazine/kg body weight, per os, in 0.1 N HCl (0.1 ml/100g body weight) or only vehicle (control). Also at time zero each rat received by intraperitoneal injection 100 uCi ^{3}H-methyl-methionine (15 Ci/mmole, Amersham Corp. Arlington

Heights, IL) in 0.1 ml 0.9% NaCl; the methionine injections were repeated hourly and the animals were decapitated one hour following the last methionine injection. At 1, 3, 5, and 9 hours after hydrazine administration three treated and three control rats were decapitated and liver DNA from pooled livers was isolated. The DNA was hydrolyzed and carrier 7-methylguanine was added to the hydrolysate before fractionation by high pressure liquid chromatography (Whatman Partisil M9 10/50 strong cation exchange column, 0.1 M ammonium phosphate pH 2.0, 4 ml/min, detection at 275 nm). The individual fractions were collected and the amount of guanine was measured by UV absorbance. Each fraction was applied to a 4 cm Dowex 50, 8x, cation exchange column previously washed with 1 N HCl. After the pyrimidine oligonucleotides or purines were absorbed onto the column, the salt was removed with 0.01 N HCl. The DNA fraction was eluted from the desalted column with 0.1 M ammonium acetate, pH 10. The eluate was neutralized with acetic acid and mixed with liquid scintillation cocktail (Aquasol 2, New England Nuclear, Boston, MA) to measure radioactivity. The amount of 7-methylguanine in the DNA was estimated by assuming the specific activity of the methylated purine was the same as the putative methyl donor, S-adenosylmethionine; the specific activity of S-adenosylmethionine was estimated according to the method of Craddock (1974). The results are shown in Figure 16. No 7-methylguanine was found in liver DNA from any of the control rats.

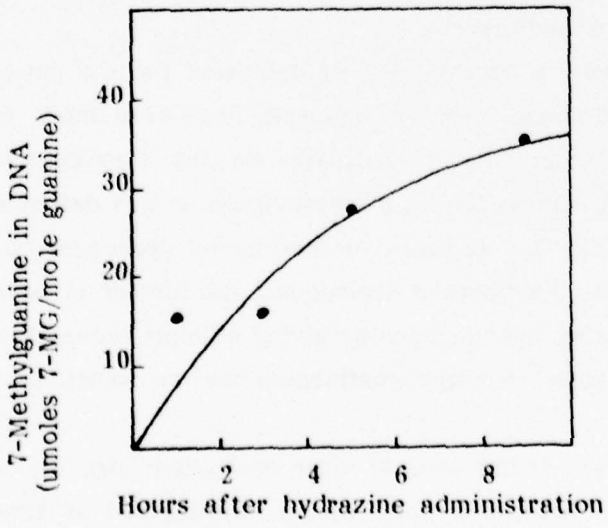


Figure 16. Time course of liver DNA methylation in rats treated with hydrazine.

The levels of DNA methylation measured using ^{3}H-methyl-methionine are approximately three times lower than those determined with the ^{14}C-labeled amino acid, at least at the 5-hour time point, the only time for which comparison data are available. This may be due to the extremely high specific activity of the tritiated

compound and therefore limited amount of methionine injected in this experiment.

One mechanism by which this methylation of DNA could occur in hydrazine-poisoned rats was suggested earlier to involve first the in vivo methylation of hydrazine to monomethylhydrazine with methionine as the source of the methyl group; monomethylhydrazine (MMH), then, might be metabolized to a methylating agent, methonium ion perhaps, thus involving the DNA. The rapid appearance of 7-methylguanine in the liver DNA of hydrazine-poisoned rats argues against monomethylhydrazine as a possible intermediate in the methylation process. Also, we previously reported (Shank, 1978) that in mice given equal molar doses of hydrazine or monomethylhydrazine, DNA methylation was only 50% greater in the monomethylhydrazine-treated animals, much less than would be expected if MMH was an intermediate in the methylation process.

The rapid methylation of DNA following oral hydrazine administration to rats is consistent with the second mechanism proposed previously (Shank, 1978): hydrazine may alter DNA methylase such that the enzyme methylates not only the 5-position of cytosine (the only methylation site in control animals) but also the 7-position of guanine. Kudrashova and Vanyushin (1976) have shown that hydrocortisone (mimicking stress) stimulates DNA methylase maximally in only 40 minutes. Our preliminary study indicated that hydrocortisone stimulation may also result in DNA methylation at the 7-position of guanine. Further studies are planned to explore the possibility that hydrazine acts by directly or indirectly altering the activity of endogenous liver DNA methylase.

One experiment has already begun but is not complete. Rats were given an LD50 dose of carbon tetrachloride, yellow phosphorus, or thioacetamide and then ^{3}H-methyl-methionine, as has been done in the hydrazine studies. Five hours after poisoning, liver DNA was isolated and analyzed for the presence of methylated bases. The object of this experiment is to determine whether DNA methylation in hydrazine-treated animals is specific to hydrazine or a non-specific response to toxic liver injury (carbon tetrachloride, phosphorus, and thioacetamide are all hepatotoxic).

MONOMETHYLHYDRAZINE METABOLISM

As explained above for hydrazine metabolism, efforts have concentrated on metabolic pathways which might involve chemical alteration of DNA and be relevant to the putative carcinogenicity of MMH. Earlier studies showed that MMH can break down or be metabolized in the rat and mouse to a methylating intermediate, resulting in the formation of 7-methylguanine in DNA. The same process was studied in the hamster. Eight young adult male Syrian golden hamsters were given orally 22 mg

MMH/kg body weight (shown previously to be an LD50) in dilute HCl. The animals developed intermittent convulsions but none died within the 5-hour study period. Five hours after MMH administration the animals were decapitated and DNA was isolated from pooled livers, kidneys, lungs, brains, and colons. Dilute HCl hydrolysates of DNA were fractionated by high pressure chromatography and examined for the presence of methylated purines by the fluorescence technique described above. No methylated purines could be detected; limits of detection were 85 umoles 7-methylguanine and 2 umoles 0^6-methylguanine per mole guanine. Thus, methylation of liver DNA in hamsters given 22 mg MMH/kg is considerably less than the 338 umoles 7-methylguanine/mole guanine reported previously for mice given 14.4 mg MMH/kg, but may be in the range of that observed in the rat given 15 mg MMH/kg, namely, 5-21 umoles 7-methylguanine/mole guanine in liver DNA.

To confirm the relatively high amount of DNA methylation in MMH-treated mice, the experiment was repeated in which mice were given 14.4 mg MMH/kg body weight and liver and lung DNA were isolated 5 hours later. Fluorescence monitoring of the effluent from the liquid chromatographic fractionation of the DNA hydrolysates failed to detect any 7-methylguanine in either DNA preparation, which suggests the earlier report of DNA methylation in MMH-treated mice was an overestimate. Hawks and Magee (1974) have shown that 15 mg MMH/kg body weight given subcutaneously to mice 6 hours before decapitation results in the formation of 63 umoles 7-methylguanine per mole guanine, a level below the limit of detection for the fluorescene technique.

Fresh MMH was synthesized using ^{14}C- or ^{3}H-dimethylsulfate to redetermine the level of DNA methylation in MMH-treated animals. In the first experiment one rat was given 0.446 mg MMH (0.0236 LD50) in 2.3 ml dilute H_2SO_4 orally at a rate of 1 ml at time zero and 1.3 ml at time one hour. The MMH was mixed to contain 228 uCi ^{3}H and 63.7 uCi ^{14}C. One rat was given an equivalent amount of unlabeled MMH. Five hours after MMH administration both rats were decapitated and DNA was isolated from pooled livers, kidneys, lungs, and colons. The DNA was hydrolyzed in 0.1 N HCl at 70°C for 30 minutes, and the hydrolysate was fractionated by high pressure liquid chromatography. Fractions containing pyrimidine oligonucleotides, guanine, adenine, 7-methylguanine, and 0^6-methylguanine (carrier added for last two bases) were desalted on Dowex 50 columns as described under hydrazine metabolism. The desalted fractions have not yet been counted for radioactivity.

Last year a series of experiments was initiated in which the in vitro oxidation of ^{14}C-MMH and ^{14}C-1,1-dimethylhydrazine (UDMH) was measured in rat liver, kidney,

lung, and colon; these experiments have been expanded to include mouse and hamster tissues and to include oxidation of ^{14}C-glucose for comparative purposes. The object of these experiments was to determine to what extent these hydrazines are metabolized as opposed to spontaneously decomposed. Earlier attempts to measure formation of formaldehyde from MMH and UDMH were not successful, presumably due to the rapid reaction of unmetabolized hydrazine with the formaldehyde to form the corresponding hydrazone; thus the formaldehyde wasn't trapped by the Nash reagent. Although the experiments are elementary, they are important; the entire thesis of the comparative metabolism program in the carcinogenicity study on the hydrazines is based on the assumption that a difference in the carcinogenicity of the propellant hydrazines in the three test species can be explained by a difference in the metabolism of these compounds in those species. If the hydrazines decompose spontaneously to the ultimate carcinogen, the likelihood is reduced that differences in metabolism will be important in explaining the differences in carcinogenicity.

Fresh tissue was sliced (0.25 mm thickness) and washed in cold saline; 200 mg of tissue slices were added to a metabolism flask containing 3 ml Krebs-Ringer phosphate buffer, pH 7.4; other slices were first boiled at 100^{o}C for one minute to serve as controls. Preincubation at 37^{o} was carried out for 10 minutes, and then 0.1 ug ^{14}C-MMH (14.6 uCi/ml, 6.6 mCi/mmole) and 2 ul containing 20 ug MMH in 0.1 N HCl were added; for UDMH 0.00135 ug ^{14}C-UDMH (18 uCi/ml, 13.3 mCi/mmole) and 2 ul containing 100 ug UDMH in 0.1 N HCl were added. These concentrations of hydrazines are approximately equivalent to the rat LD_{50} ip or po, assuming that 10% of the dose reaches the tissue at one time. Carbon dioxide was trapped in the center well by NaOH. After the appropriate incubation time, the tissue slices were inactivated by the addition of 6 ml cold absolute ethanol. Sodium carbonate was added to the NaOH as carrier and the carbonate was precipitated as the barium salt. The barium carbonate was washed, boiled in water, and counted for ^{14}C; in this way the ^{14}C represented only CO_2 and not entrapped MMH or UDMH. The filtered incubation medium was also assayed for ^{14}C as a check on how much ^{14}C was added to the flask initially.

The results for the oxidation of MMH are summarized in Table 3 and Figures 17-19. The table gives regression lines calculated for the data over the 240-minute incubation period; however, in most instances a biphasic response was observed, showing a greater rate of oxidation occurring in the first 60 minutes of incubation. The biphasic response is made clear in the graphic representation of the experimental data in Figures 17-19.

TABLE 3

LINEAR REGRESSION ANALYSIS FOR IN VITRO METABOLIC OXIDATION OF ^{14}C-MMH TO $^{14}CO_2$ BY RAT, MOUSE, AND HAMSTER TISSUE SLICES IN FOUR HOURS AT 37°C

Species	Tissue	Regression Line*	Correl. Coef.
Rat	Liver	$y = 0.030x - 0.36$	0.95
	Kidney	$y = 0.012x + 0.21$	0.95
	Colon	$y = 0.011x + 0.14$	0.87
	Lung	$y = 0.004x + 0.01$	0.99
Mouse	Liver	$y = 0.035x + 0.24$	0.87
	Kidney	$y = 0.017x + 0.61$	0.78
	Colon	$y = 0.010x - 0.06$	0.92
	Lung	$y = 0.007x + 0.034$	0.97
Hamster	Liver	$y = 0.0340x + 0.2116$	0.72
	Kidney	$y = 0.0093x + 0.1233$	0.91
	Colon	$y = 0.0064x + 0.1362$	0.84
	Lung	$y = 0.0040x + 0.1055$	0.79

* y = percentage administered radioactivity recovered as carbon dioxide

x = incubation time in miutes, up to 240 minutes

In light of the fact that formaldehyde formation could not be measured in the presence of MMH, presumably due to hydrazone formation by the formaldehyde and MMH, it is surprising that so much $^{14}CO_2$ could be recovered in these tests. It may be possible that in the first hour some of the MMH may be oxidatively demethylated and the resulting formaldehyde forms the hydrazone with MMH, while MMH also breaks down spontaneously to a product which is rapidly oxidized to carbon dioxide; after the first hour the spontaneous breakdown products could be gone and one then measures the rate of the oxidative demethylation of the hydrazone which formed earlier. This is only one of several possible explanations for the biphasic curve; no further effort is planned to characterize the intermediates between MMH and carbon dioxide in these experiments.

The objective of the study was achieved; it has been demonstrated clearly that

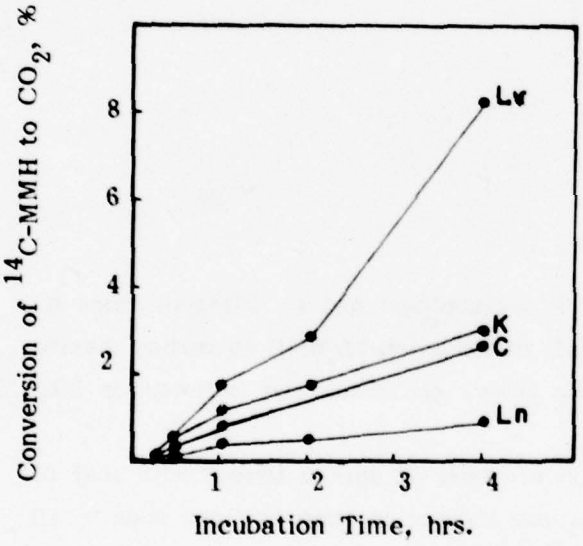


Figure 17. In vitro oxidation of ^{14}C-MMH to $^{14}CO_2$ by rat liver (Lv), kidney (K), colon (C), and lung (Ln) slices at 37°C. Fischer 344 young male rats; each point represents triplicate assays.

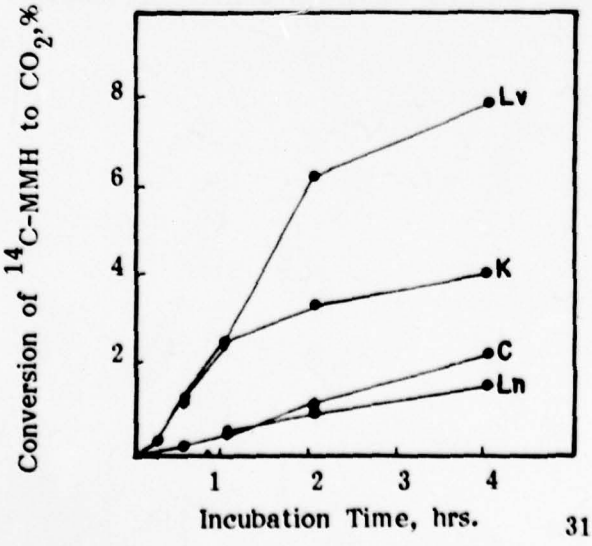


Figure 18. In vitro oxidation of ^{14}C-MMH to $^{14}CO_2$ by mouse liver (Lv), kidney (K), colon (C), and lung (Ln) slices at 37°C. C57Bl/6 young male mice; each point represents triplicate assays.

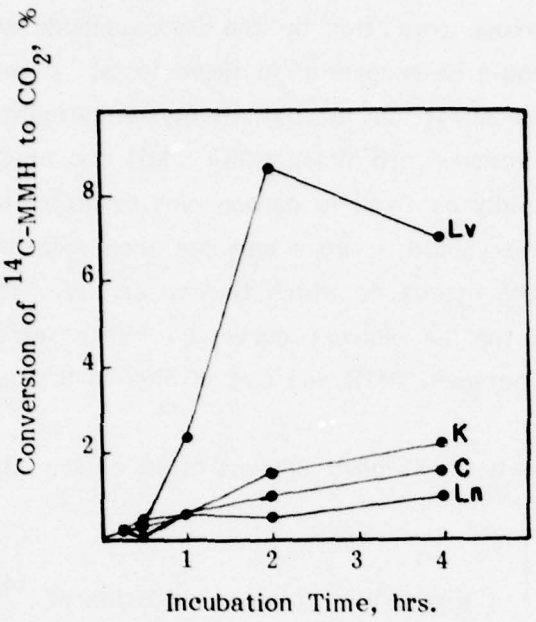


Figure 19. In vitro oxidation of ^{14}C-MMH to $^{14}CO_2$ by hamster liver (Lv), kidney (K), colon (C), and lung (Ln) slices at 37°C. Syrian golden young male hamsters; each point represents triplicate assays.

under these experimental conditions, MMH is metabolized and at different rates by different tissues in the three species. The rate of oxidation of MMH to carbon dioxide is greatest in the liver and decreases in the kidney and colon and is lowest in lung (Figures 17-19).

Table 4 compares the rate of oxidation of MMH by animal tissues with that of glucose under the same experimental conditions; incubation time was one hour in all cases. The rat appears to be able to oxidize MMH about as rapidly as glucose. On the other hand most mouse and hamster tissues, with the exception of liver, are slower to oxidize MMH.

TABLE 4

COMPARISON OF THE RATES OF METABOLIC OXIDATION OF ^{14}C-MMH AND ^{14}C-GLUCOSE TO $^{14}CO_2$ BY RAT, MOUSE, AND HAMSTER TISSUE SLICES AT 37°C FOR ONE HOUR.

		Conversion of Substrate to $^{14}CO_2$ in 1 Hour, %			
Species	Substrate	Liver	Kidney	Colon	Lung
Rat	MMH	1.80	1.23	0.82	0.35
	Glucose	1.21	2.41	1.54	0.65
Mouse	MMH	2.55	2.66	0.41	0.49
	Glucose	0.09	4.40	1.23	2.03
Hamster	MMH	2.29	0.55	0.43	0.63
	Glucose	0.06	2.41	9.98	1.23

Figures 20-23 compare the rates of metabolic oxidation of ^{14}C-MMH to $^{14}CO_2$ for each tissue in rat, mouse, and hamster.

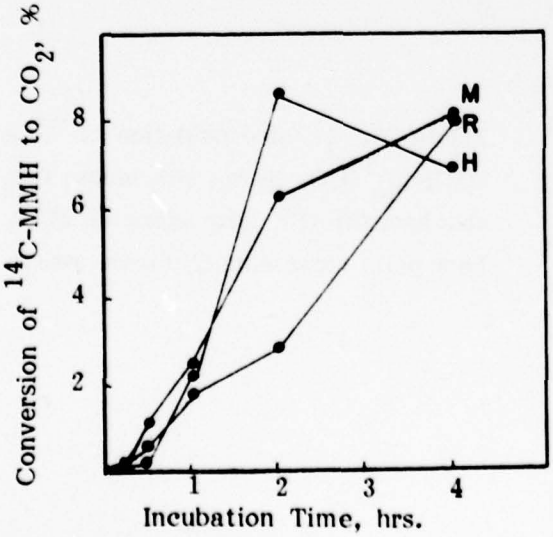


Figure 20. In vitro oxidation of ^{14}C-MMH to $^{14}CO_2$ by rat (R), mouse (M), and hamster (H) liver slices at 37°C. Each point represents triplicate assays.

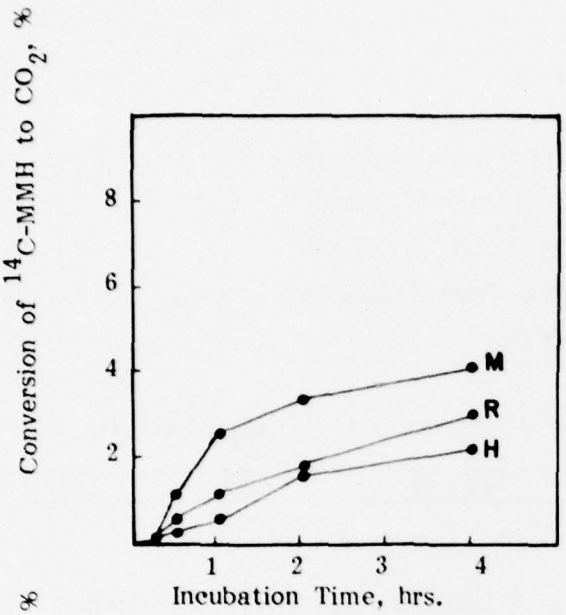


Figure 21. In vitro oxidation of ^{14}C-MMH to $^{14}CO_2$ by rat (R), mouse (M), and hamster (H) kidney slices at 37°C. Each point represents triplicate assays.

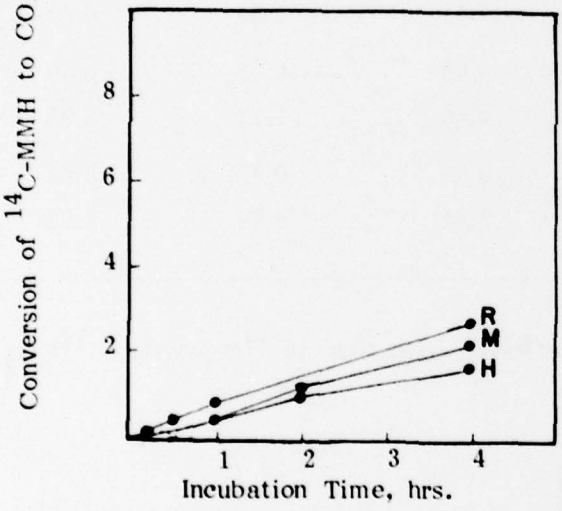


Figure 22. In vitro oxidation of ^{14}C-MMH to $^{14}CO_2$ by rat (R), mouse (M), and hamster (M) colon slices at 37°C. Each point represents triplicate assays.

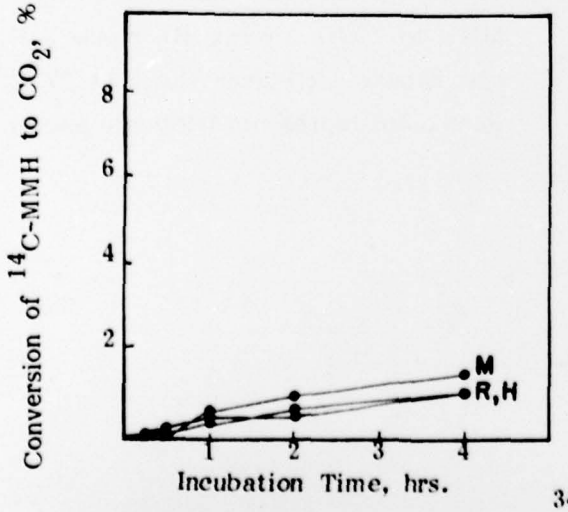


Figure 23. In vitro oxidation of ^{14}C-MMH to $^{14}CO_2$ by rat (R), mouse (M), and hamster (H), lung slices at 37°C. Each point represents triplicate assays.

From these figures it is clear that although mouse liver oxidizes MMH to CO_2 faster than rat or hamster liver, the end point after 4 hours incubation is about the same in all three species. It is not yet clear how the faster oxidation of MMH in mouse liver is related to the greater methylation of mouse liver DNA compared to rat liver DNA. Mouse kidney converts MMH to CO_2 faster than rat kidney which is faster than hamster kidney, and colon and lung are slow converters in all three species.

1,1-DIMETHYLHYDRAZINE METABOLISM

The metabolism studies on 1,1-dimethylhydrazine (UDMH) have paralleled those on MMH. Rats and hamsters were given LD50 doses of UDMH orally and decapitated 5 hours later. DNA was isolated from liver, kidney, lung, colon, and brain, hydrolyzed in 0.1 N HCl at 70°C for 30 minutes, and analyzed by high pressure liquid chromatography with a fluorescence detector; no methylated purines could be detected with this technique which has limits of detection of 85 umoles 7-methylguanine per mole guanine and 2 umoles 0^6-methylguanine per mole guanine.

Before the DNA alkylation studies on UDMH could be done in the mouse, it was necessary to estimate the LD50 value in the C57/Bl/6 mouse for a 5-hour duration. Mice were given orally 150, 175, 200 or 265 mg UDMH/kg body weight (UDMH was dissolved in 0.1 N HCl) and observed for 5 hours. The number of animals that died at each dose is given in Table 5.

TABLE 5

ACUTE LETHALITY OF UDMH IN THE C57BL/6 MOUSE

Dose UDMH, po mg/kg body wt.	No. Mice Tested	No. Mice Dead in 5 Hours	% Dead
150	18	4	22
175	19	7	37
200	14	8	57
265	21	19	90.5

LD50=190 mg/kg BW 95%CL=171-211 mg/kg BW S=1.30

The LD50 with 95% confidence limits and slope function, S, for the 5-hour observation period was calculated according to the method of Litchfield and Wilcoxon (1949).

At the end of 5 hours, survivors of the LD50 determination were decapitated and DNA was isolated from livers, kidneys and lungs. Analysis by high-pressure liquid chromatography and fluorescence spectrometry failed to detect the presence of 7-methylguanine or 0^6-methylguanine in the DNA from animals treated with any of the four doses of UDMH.

Obviously, if UDMH administration results in any DNA methylation of these tissues in rats, mice, or hamsters, the extent of methylation must be quite small and radiolabeled UDMH will have to be used to detect the DNA adduct.

Radioactive UDMH was prepared in the laboratory and administered orally to one Fischer 344 male rat. A mixture of ^{14}C- and ^{3}H-labeled UDMH was used to determine if the two labeled compounds would give identic[illegible] results; earlier work with ^{3}H-UDMH indicated the label on DNA was bound sufficiently strong to withstand the DNA isolation technique but not the 0.1 N HCl hydrolysis. The rat received 0.604 mg ^{14}C-UDMH (132 uCi; 13.3 mCi/mmole) and 4.2 ug ^{3}H-UDMH (280 uCi; 122 mCi/mmole) in 2.2 ml diluted H_2S0_4 (pH 1) divided into two intubations given an hour apart. One additional rat was given the same amount of unlabeled UDMH. Five hours after the first administration of UDMH, the rats were decapitated and DNA was isolated from individual livers and pooled kidneys, lungs, and colons. These nucleic acid specimens have not yet been analyzed by high pressure liquid chromatography (reverse phase) and liquid scintillation spectrometry.

To confirm that UDMH undergoes metabolism as opposed to spontaneous decomposition in rodent tissues, the rate of oxidation of ^{14}C-UDMH to $^{14}CO_2$ was measured using tissue slices. The technique used was described in the study on the in vitro metabolic oxidation of ^{14}C-MMH. The results for the oxidation of UDMH are summarized in Table 6 and Figures 24-26.

TABLE 6

LINEAR REGRESSION ANALYSIS FOR IN VITRO METABOLIC OXIDATION OF ^{14}C-UDMH TO $^{14}CO_2$ BY RAT, MOUSE, AND HAMSTER TISSUE SLICES IN FOUR HOURS AT 37°C

Species	Tissue	Regression Line*	Correl. Coeff.
Rat	Liver	$y = 0.01x - 0.02$	0.96
	Kidney	$y = 0.0072x + 0.085$	0.96
	Colon	$y = 0.008x + 0.17$	0.84
	Lung	$y = 0.0008x + 0.0006$	0.91
Mouse	Liver	$y = 0.008x + 0.48$	0.74
	Kidney	$y = 0.009x + 0.46$	0.69
	Colon	$y = 0.002x + 0.002$	0.96
	Lung	$y = 0.011x + 0.065$	0.93
Hamster	Liver	$y = 0.0119x + 0.0514$	0.95
	Kidney	$y = 0.0051x + 0.0393$	0.82
	Colon	$y = 0.0028x + 0.0284$	0.34
	Lung	$y = 0.0003x + 0.0091$	0.44

* y = percentage administered radioactivity recoverd as carbon dioxide

x = incubation time in minutes, up to 240 minutes

The table gives regression lines calculated for the data over the 240-minute incubation period. In most instances, however, a biphasic response was seen, with a greater rate of oxidation occurring in the first hour of incubation. The biphasic response can be seen in Figures 24-26. As was the case for MMH, the nature of this response cannot yet be explained. This study does demonstrate that UDMH is metabolized by these tissues in vitro and at different rates in the three species. For example, mouse lung is considerably more active in oxidizing UDMH than is lung from the rat or hamster; in fact, mouse lung is just as active as rat and hamster liver. In general, rodent tissues oxidized MMH faster than UDMH.

Table 7 compares the rate of oxidation of UDMH by animal tissues with that of glucose under the same experimental conditions, and thus, compares with Table 4 which provides the same information for MMH; incubation time was one hour in all cases.

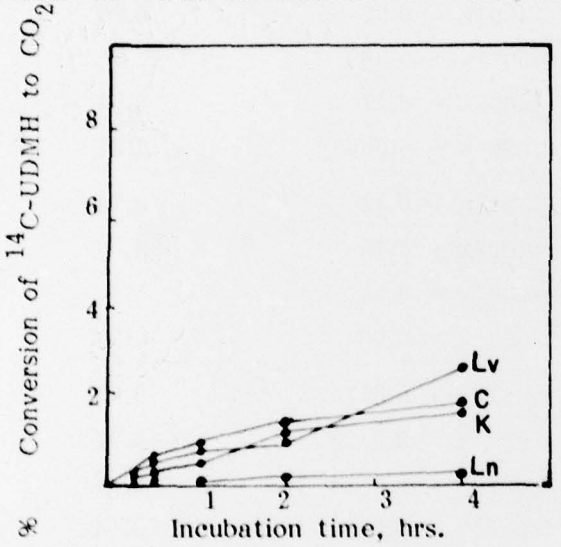


Figure 24. In vitro oxidation of ^{14}C-UDMH to $^{14}CO_2$ by rat liver (Lv), kidney (K), colon (C), and lung (Ln) slices at 37°C. Fischer 344 young male rats; each point represents triplicate assays.

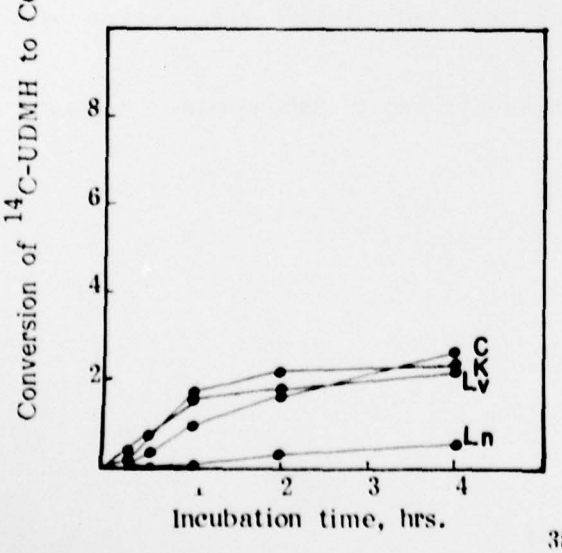


Figure 25. In vitro oxidation of ^{14}C-UDMH to $^{14}CO_2$ by mouse liver (Lv), kidney (K), colon (C), and lung (Ln) slices at 37°C. C57Bl/6 young male mice; each point represents triplicate assays.

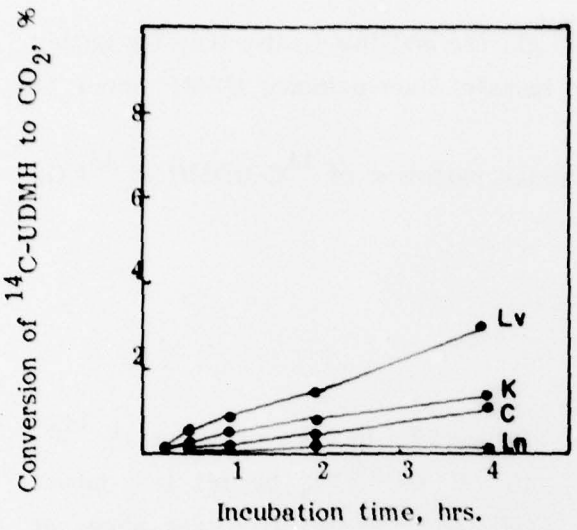


Figure 26. In vitro oxidation of ^{14}C-UDMH to $^{14}CO_2$ by hamster liver (Lv), kidney (K), colon (C), and lung (Ln) slices at 37°C. Syrian golden young male hamsters; each point represents triplicate assays.

TABLE 7

COMPARISON OF THE RATES OF METABOLIC OXIDATION OF ^{14}C-UDMH AND ^{14}C-GLUCOSE TO $^{14}CO_2$ BY RAT, MOUSE, AND HAMSTER TISSUE SLICES AT 37°C FOR ONE HOUR

		Conversion of Substrate to $^{14}CO_2$ in 1 hr., %			
Species	Substrate	Liver	Kidney	Colon	Lung
Rat	UDMH	0.70	0.54	0.84	0.04
	Glucose	1.21	2.41	1.54	0.65
Mouse	UDMH	1.59	1.77	0.97	0.12
	Glucose	0.09	4.40	1.23	2.03
Hamster	UDMH	0.90	0.52	0.24	0.10
	Glucose	0.06	2.41	9.98	1.23

All rat tissues oxidized UDMH slower than glucose and this is also true for kidney and lung from mice and hamsters. Mouse and hamster liver oxidized UDMH about 15 times faster than glucose.

Figures 27-30 compare the rates of metabolic oxidation of ^{14}C-UDMH to $^{14}CO_2$ for each tissue in rat, mouse, and hamster.

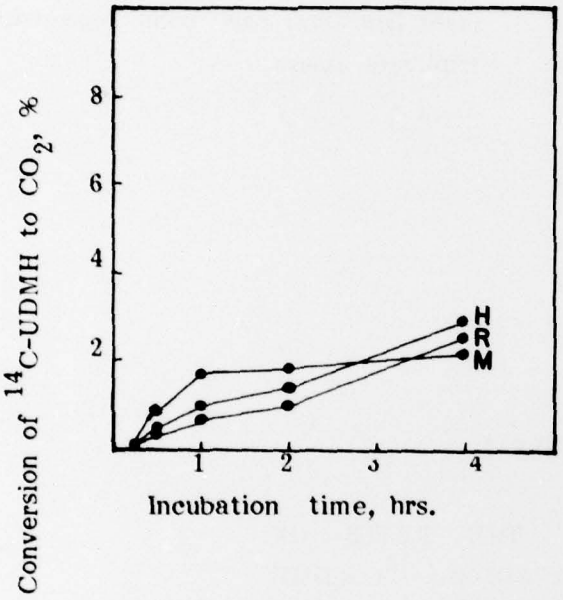


Figure 27. In vitro oxidation of ^{14}C-UDMH to $^{14}CO_2$ by rat (R), mouse (M), and hamster (H) liver slices at 37°C. Each point represents triplicate assays.

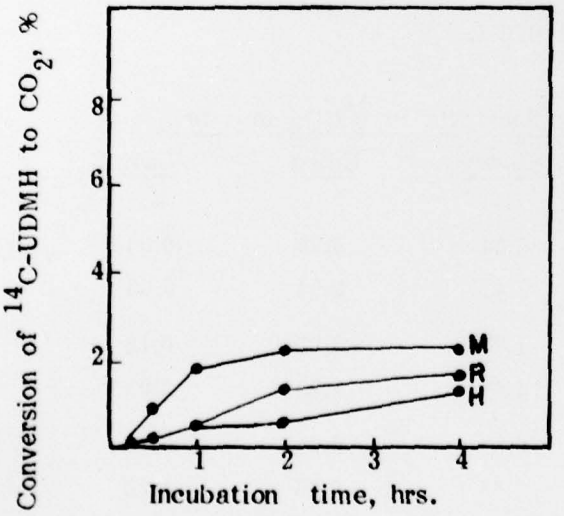


Figure 28. In vitro oxidation of ^{14}C-UDMH to $^{14}CO_2$ by rat (R), mouse (M), and hamster (H) kidney slices at 37°C. Each point represents triplicate assays.

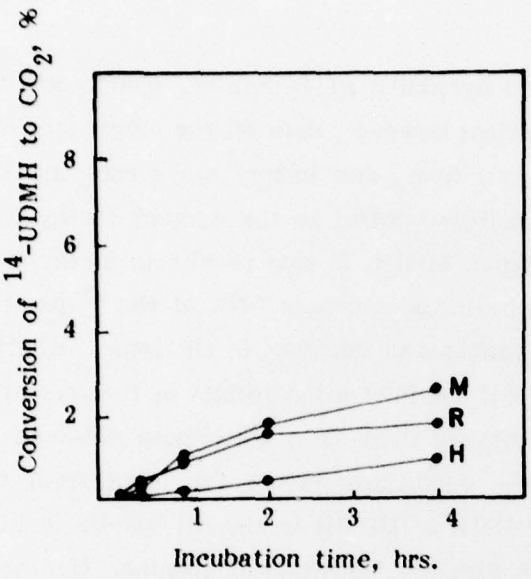


Figure 29. In vitro oxidation of ^{14}C-UDMH to $^{14}CO_2$ by rat (R), mouse (M), and hamster (H) colon slices at 37°C. Each point represents triplicate assays.

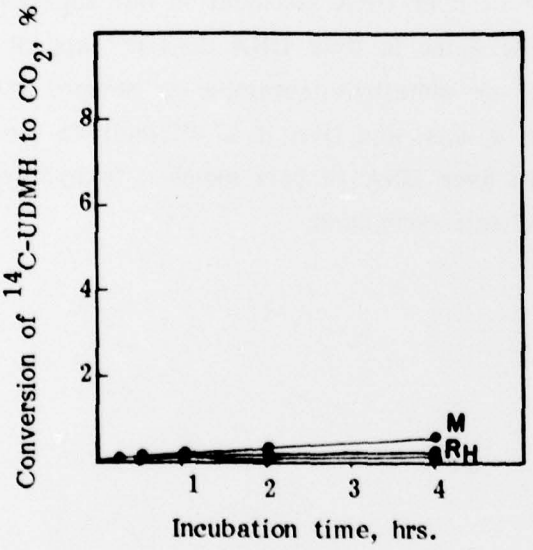


Figure 30. In vitro oxidation of ^{14}C-UDMH to $^{14}CO_2$ by rat (R), mouse (M), and hamster (H) lung slices at 37°C. Each point represents triplicate assays.

From these figures it can be seen that mouse liver oxidizes UDMH to CO_2 faster than does rat or hamster liver, although the total oxidation over the 4-hour incubation period is the same for rat, mouse, and hamster liver; this pattern in hepatic metabolism of UDMH was also seen in a similar study on MMH. The mouse kidney oxidizes UDMH faster than does rat or hamster kidney, as was also seen in the MMH studies. None of the lung slices from any of the three species metabolized UDMH to CO_2 to an appreciable extent. If indeed UDMH is carcinogenic to the mouse lung, as believed by Toth (1973), then the metabolic activation of UDMH in mouse lung would seem not to correlate with metabolic oxidation of UDMH to CO_2.

1,2-DIMETHYLHYDRAZINE METABOLISM

The data so far available on the carcinogenicity of hydrazine, monomethylhydrazine, and 1,1-dimethylhydrazine lack definition; however, data on the carcinogenicity of 1,2-dimethylhydrazine (SDMH) in the colon, liver, and kidney are clear, and this compound, SDMH, has been selected as a positive control in the current methylation studies. Administration of the strong carcinogen, SDMH, to rats results in methylation of liver, colon, and kidney DNA but not lung, brain, or pancreas DNA at the 0^6-position of guanine and, in some cases, the 7- and 3-positions of adenine; in all tissues, whether a target organ for carcinogenicity or not, SDMH administration results in the formation of 7-methylguanine in the DNA. Table 8 compares these data with those obtained for hydrazine, MMH, and UDMH under the same conditions except for dose; from this comparison it is clear that administration of MMH or UDMH to the rat results in little DNA methylation, even at the most accessible site, the 7-position of guanine. Hydrazine administration does result in a small amount of liver DNA methylation but apparently only at the 7-position of guanine. 7-Methylguanine in liver DNA does not appear to be related to the carcinogenicity of SDMH or dimethylnitrosamine or several other carcinogens which are potential methylating agents, and thus it is difficult to see at this time how 7-methylguanine formation in liver DNA of rats exposed to hydrazine is related to the putative carcinogenicity of this compound.

TABLE 8

IN VIVO DNA METHYLATION IN RATS TREATED WITH HYDRAZINE, MONOMETHYLHYDRAZINE (MMH), 1,1-DIMETHYLHYDRAZINE (UDMH), AND 1,2-DIMETHYLHYDRAZINE (SDMH)

Agent	Dose (mg/kg BW)	DNA Source	Target*	Methylated Purines, umoles/mole guanine**		
				7MG	0^6MG	Others
Hydrazine	60	liver	-	150-196	ND	ND
	30	liver	-	ND	ND	ND
	60	lung	-	ND	ND	ND
	60	kidney	-	ND	ND	ND
MMH	15	liver	-	5-21	ND	ND
	15	kidney	-	ND	ND	ND
	15	lung	-	ND	ND	ND
	15	brain	-	ND	ND	ND
	15	colon	-	ND	ND	ND
UDMH	6	liver	(+)	ND	ND	ND
	6	kidney	-	ND	ND	ND
	6	lung	-	ND	ND	ND
	6	colon	-	ND	ND	ND
SDMH	10	liver	(+)	1716	332	ND
	10	colon	+	103	36	ND
	10	kidney	(+)	trace	12	ND
	160	liver	(+)	2450	264	7MA,3MA
	160	colon	+	548	55	3MA
	160	kidney	(+)	101	11	3MA
	160	brain	-	148	ND	ND
	160	lung	-	57	ND	ND
	160	pancreas	-	50	ND	ND

* target = organ (referred to under DNA Source) in which tumor is produced; (+) weakly carcinogenic to this organ.

** 7MG = 7-methylguanine; 0^6MG = 0^6-methylguanine; 7MA = 7-methyladenine; 3MA = 3-methyladenine.

SUMMARY AND CONCLUSIONS

Considerable advances have been made in developing new techniques to facilitate the studies on the comparative metabolism of the propellant hydrazines. A high pressure liquid chromatographic method to analyze DNA hydrolysates has been developed and has limits of sensitivity as low as 85 umoles 7-methylguanine and 2 umoles 0^6-methylguanine per mole guanine. At this sensitivity methylation can be quantitated in as little as 5 ug DNA, permitting analysis in a small organ (lung) from a single animal, not possible using radiolabel techniques.

Levels of DNA methylation in animals treated with the propellant hydrazine have been shown to be below these limits of detection, thus necessitating use of radiolabels and analysis of large amounts of purified DNA. Conventional methods for chromatographic analysis of such large amounts of DNA (10-100 mg) require the use of inorganic mobile phases which present several problems in the measurement of radioactivity. These problems have been circumvented by the development of a reverse phase chromatographic fractionation of DNA hydrolysates which is salt-free. This permits ready analysis for ^{14}C and ^{3}H, and also facilitates derivatization of eluted pyrimidines and purines, such as silylation for GC/MS analysis.

In fact 10 pyrimidines and purines have been silylated and their mass spectra obtained for use in confirmation of identification of abnormal bases in DNA. Mass spectra have been obtained on silylated derivatives of a) the normal DNA pyrimidines, thymine, cytosine, and 5-methylcytosine, b) the RNA pyrimidine, uracil, which would represent RNA contamination of the DNA, c) the normal DNA purines, guanine and adenine, and d) the abnormal alkylated purines, 7-methylguanine, 0^6-methylguanine, 0^6-ethylguanine, and N^6-methyladenine.

Final preparation has begun for the study of the fate of the nitrogen atoms of hydrazine, monomethylhydrazine, and 1,1-dimethylhydrazine in relation to possible adduct with DNA. DNA is isolated from animals treated with ^{15}N-labeled hydrazines, digested to convert the nitrogen to ammonia which is then distilled into an excess of ethyliodide to form mono-, di-, and tri-ethylamine. The amines are identified and quantitated by GC/MS. If it can be shown that appreciable ^{15}N from the hydrazines adducts with DNA, the studies will procede to analyze DNA hydrolysates by high pressure liquid chromatography and GC/MS to determine with which pyrimidines and purines the ^{15}N is associated.

Studies on the metabolism of hydrazine have continued. The formation of 7-methylguanine in liver DNA of rats and mice treated with hydrazine has been confirmed and quantitated. Methylation levels are at the limit of detection by the optical (fluorescence) technique and therefore radiolabel experiments must be continued. Tritiated methionine can be used in place of the ^{14}C-labeled amino acid, which will reduce the expense of these studies. Methylation of DNA appears to be limited to the 7-position of guanine; 0^6-methylguanine has been seen in liver DNA of hydrazine-poisoned mice but this response is capricious. In the rat, 7-methylguanine forms rapidly which argues against the suggestion that hydrazine may first be methylated to form MMH which then is converted to an active methylating agent. The rapid methylation of liver DNA in hydrazine-treated rats is comparable to the rate at which DNA is methylated in hydrocortisone-treated rats. Studies are in progress to see if this DNA methylation is specific to hydrazine insult or if this is a non-specific response to toxic liver injury.

Levels of DNA methylation following MMH treatment are low, below 80 umoles 7-methylguanine per mole guanine; this is less than that reported earlier, which was probably an overestimate. A double label experiment is in progress in which animals have been given ^{14}C- and ^{3}H-MMH; if both isotopes indicate the same level of DNA methylation, future studies will use ^{3}H-MMH. DNA methylation following UDMH has not been demonstrated.

Methylation of DNA by a strong carcinogen, SDMH, is much more extensive than with MMH (or UDMH). Following SDMH administration, 7-methlyguanine can be found in liver, kidney, colon, lung, brain, and pancreas (all the tissues examined so far) and other methylated bases are seen in the target organs, colon, liver, and kidney. MMH administration results in only 7-methylguanine formation and that is limited to only liver, not a target organ. Such limited alkylation is not expected if the mechanism of action of MMH carcinogenicity is similar to that of SDMH.

Earlier studies left some doubt as to whether MMH and UDMH were metabolized at all in in vitro systems. We have now confirmed that these compounds can be metabolically oxidized to CO_2, that different species and different tissues have different rates of oxidation; generally MMH is oxidized faster than UDMH, that liver is faster than kidney which is faster than colon and lung, and that mouse liver and kidney oxidize MMH and UDMH rapidly relative to rates observed with rat and hamster tissues.

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